Exhibit 3

...LARATION OF PAUL POLAKIS, Ph.D.

I, P... h.D., declare and say as follows:

1. I we... Ph.D. by the Department of Biochemistry of the Michigan State Univ... 4. My scientific Curriculum Vitae is attached to and forms part of this ... (Exhibit A).

2. I am... ...ployed by Genentech, Inc. where my job title is Staff Scientist. ... ...enentech in 1999, one of my primary responsibilities has been leadin... ...'s Tumor Antigen Project, which is a large research project with a prim... ...dentifying tumor cell markers that find use as targets for both the di... ...reatment of cancer in humans.

3. As p... ...or Antigen Project, my laboratory has been analyzing different... ...arious genes in tumor cells relative to normal cells. The purpos... ...h is to identify proteins that are abundantly expressed on certain ... ...hat are either (i) not expressed, or (ii) expressed at lower leve... ...ing normal cells. We call such differentially expressed proteins "... ...teins". When such a tumor antigen protein is identified, e... ...an antibody that recognizes and binds to that protein. Such an ant... ...in the diagnosis of human cancer and may ultimately serve as ... ...utic in the treatment of human cancer.

4. I... ...search conducted by Genentech's Tumor Antigen Project, ...a variety of scientific techniques for detecting and studyin... ...pression in human tumor cells relative to normal cells, at genom... ...d protein levels. An important example of one such techniqu... ...and widely used technique of microarray analysis which h... ...mely useful for the identification of mRNA molecules that are ... ...sed in one tissue or cell type relative to another. In the course o... ...microarray analysis, we have identified approxim... ...scripts that are present in human tumor cells at significa... ...n in corresponding normal human cells. To date, we have ge... ...t bind to about 30 of the tumor antigen proteins expresse... ...ially expressed gene transcripts and have used these antibodi... ...etermine the level of production of these tumor antigen ... ...n cancer cells and corresponding normal cells. We have the... ...s of mRNA and protein in both the tumor and normal cells an...

5. F... ...rotein expression analyses described in paragraph 4 above, w... ...here is a strong correlation between changes in the level of ... ...particular cell type and the level of protein

expres[illegible] in that cell type. In approximately 80% of our observ[illegible] that increases in the level of a particular mRNA correla[illegible] the level of protein expressed from that mRNA when human t[illegible] mpared with their corresponding normal cells.

6. [illegible] experience accumulated in more than 20 years of researc[illegible] discussed in paragraphs 4 and 5 above and my knowl[illegible] cientific literature, it is my considered scientific opinio[illegible] s, an increased level of mRNA in a tumor cell relative to a no[illegible] correlates to a similar increase in abundance of the encode[illegible] cell relative to the normal cell. In fact, it remains a central [illegible] biology that increased mRNA levels are predictive of corresp[illegible] els of the encoded protein. While there have been publish[illegible] r which such a correlation does not exist, it is my opinio[illegible] exceptions to the commonly understood general rule that inc[illegible] are predictive of corresponding increased levels of the encode[illegible]

7. [illegible] ll statements made herein of my own knowledge are true an[illegible] ade on information or belief are believed to be true, and fur[illegible] nts were made with the knowledge that willful false statem[illegible] de are punishable by fine or imprisonment, or both, under [illegible] 3 of the United States Code and that such willful statem[illegible] e validity of the application or any patent issued thereon.

Dated: 5.

By: Paul Polakis

Paul Polakis, Ph.D.

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JRRICULUM VITAE

PAUL G. POLAKIS
Staff Scientist
Genentech, Inc
1 DNA Way, MS#40
San Francisco, CA 94080

EDUCATION:

Ph.D., Biochemist iochemistry,
Michigan State Un

B.S., Biology. Coll ince, Michigan State University (1977)

PROFESSIONAL

2002-present	Staff Scientist, Genentech, Inc S. San Francisco, CA
1999- 2002	Senior Scientist, Genentech, Inc., S. San Francisco, CA
1997 -1999	Research Director Onyx Pharmaceuticals, Richmond, CA
1992- 1996	Senior Scientist, Project Leader, Onyx Pharmaceuticals, Richmond, CA
1991-1992	Senior Scientist, Chiron Corporation, Emeryville, CA.
1989-1991	Scientist, Cetus Corporation, Emeryville CA.
1987-1989	Postdoctoral Research Associate, Genentech, c., South SanFrancisco, CA.
1985-1987	Postdoctoral Research Associate, Department of Medicine, Duke University Medical Center, Durham, NC

1984-1985	Assistant Professor, Department of Chemistry, Oberlin College, Oberlin, Ohio
1980-1984	Graduate Research Assistant, Department of Biochemistry, Michigan State University East Lansing, Michigan

PUBLICATIONS:

1. **Polakis, P G.** and Wilson, J. E. 1982 Purification of a Highly Bindable Rat Brain Hexokinase by High Performance Liquid Chromatography. **Biochem. Biophys. Res. Commun.** 107, 937-943.

2. **Polakis, P.G.** and Wilson, J. E. 1984 Proteolytic Dissection of Rat Brain Hexokinase: Determination of the Cleavage Pattern during Limited Digestion with Trypsin. **Arch. Biochem. Biophys.** 234, 341-352.

3. **Polakis, P. G.** and Wilson, J. E. 1985 An Intact Hydrophobic N-Terminal Sequence is Required for the Binding of Rat Brain Hexokinase to Mitochondria. **Arch. Biochem. Biophys.** 236, 328-3.

4. Uhing, R.J., Polakis, P.G. and Snyderman, R. 1987 Isolaton of GTP-binding Proteins from Myeloid HL60 Cells. **J. Biol. Chem.** 262, 15575-15579.

5. **Polakis, P.G.**, Uhing, R.J. and Snyderman, R. 1988 The Formylpeptide Chemoattractant Receptor Coupled with a GTP-binding Protein Containing a Distinct 40 kDa Pertussis Toxin Substrate. **J. Biol. Chem.** 263, 4969-4979.

6. Uhing, R. J., Dillon, S., **Polakis, P.**, Truett, A. P. and Snyderman, R. 1988 Chemoattractant Receptors and Transduction Processes in Cellular and Molecular Aspects of Inflammation. (Proste, G. and Crooke, S. T. eds.) pp 335-379.

7. **Polakis, P.G.**, Evans, T. and Snyderman 1989 Multiple Chromatographic Forms of the Formylpeptide Chemoattractant Receptor and their Relationship to GTP-binding Proteins. **Biochem. Biophys. Res. Commun.** 161, 276-283.

8. **Polakis, P. G.**, Snyderman, R. Evans, T. 1989 Characterization of G25K, a GTP-binding Protein Containing a Putative Nucleotide Binding Domain. **Biochem. Biophys. Res. Commun.** , 25-32.

9. **Polakis, P.**, Wilson, R.F., Newsbury, J. Evans,T. and Snyderman, R. 1989 Identification of Neutral and Gene Products, Low Molecular Mass GTP-binding Proteins from Human Pl. **J. Biol. Chem.** 264, 16383-16389.

10. Snyderman, R., Uhing, A. T., **Polakis, P.** and Didsbury, J. 1989 G Proteins and Neutrophil Function: ADP-Ribosylating Toxins and G Proteins: Insights into Signal Transduction. (Iglewski, B. and M. Vaughn, eds.) Amer. Soc. Microbiol. pp. 29

11. Hart, M.J., Pola.. , **P.G., E** . and Cerrione, R.A. 1990 **The Identification and Charaterizatio an Epide rowth Factor-Stimulated Phosphorylation of a Specific Low** Molec Mass G ding Protein in a Reconstituted Phospholipid **Vesicle System. J. Li .. Chem.** 5990-6001.

12. Yatani, A., Okab K., **Polak** Halenbeck, R. McCormick, F. and Brown, A. **M. 1990 ras p21 an AP Inhib** pling of Muscarinic Receptors to Atrial K^+ **Channels. Cell.** 9-776.

13. Munemitsu, S., I s, **M.A.,** .., McCormick, F., Ullrich, A. and **Polakis, P.G. 1990** Molec la. oning ar ession of a G25K cDNA, the Human Homolog **of the Yeast** Cell Cy Gene Ct **Mol. Cell. Biol. 10, 5977-5982.**

14. Polakis, P.G nfeld, B ; T. and McCormick, F. 1991 **Purification of Plasma** Membr ociated e Activating Protein (GAP) Specific for rap-**1/krev-1** from HL 6 s. **Proc.** cad. **Sci. USA 88, 239-243.**

15. Moran, M. F. is, **P.,** ck, F., Pawson, T. and Ellis, C. **1991 Protein Tyrosine** Kinase F late th horylation, Protein Interactions, Subcellular **Distribution,** and Ac y of p21 Pase Activating Protein. **Mol. Cell. Biol. 11, 1804-1812**

16. Rubinfeld, B. W , G., Be Wood, A. McCormick, F. and **Polakis, P. G. 1991 A** Synthetic Pe e Corre g to a Sequence in the GTPase Activating **Protein** Inhibits p2 imulati romotes Guanine Nucleotide Exchange. **Internatl. J.** Pe l Prot. , 47-53.

17. Rubinfeld, B., M mitsu, S , R., Conroy, L., Watt, K., Crosier, W., **McCormick,** F., a u akis, P. olecular Cloning of a GTPase Activating **Protein** Specific Krev-1 F p21rap1. **Cell 65, 1033-1042.**

18. Zhang, K. P e, A., n, P., Vass, W. C., Olah, Z., **Polakis, P., McCormick,** F. D, R. terogenous Amino Acids in RAS and **Rap1A** Specifyi ivity to teins. **Science 254, 1630-1634.**

19. Martin, G., Y ., Clark kis, P., Brown, A. M. and McCormick, F. **1992 GAP** Dom ponsib 1ras-dependent Inhibition of Muscarinic Atrial **K^+ Channel** Curr cience 2-194.

20. McCormick, , G. A ., Bollag, G. and **Polakis, P . 1992 Regulation** of p2 GTPa g Proteins. Cold Spring Harbor **Symposia on Quantitative** Vol. 241.

21. Pronk, G. B., P., V deVries-Smits, A. M., Bos J. L. and **McCormick,** F. 1 -src C. ate with and Phosphorylate the $p21^{ras}$ **GTPase** Activati n. On ,389-394.

22. Polakis P. nici teractions Between $p21^{ras}$ **Proteins and Their GTPase** A Protei cer Surveys (Franks, L. M., ed.) 12, 25-**42.**

23. Wong, G., M. Clark roy, L., Moran, M., **Polakis,P. and** McCormick, F. 19 cular and nucleic acid binding properties of the GAP-associated hos p62. **Cell** 69, 551-558.

24. Polakis, P., F B: rmick, F. 1992 Phosphorylation of rap1GAP in vivo and by cA nde: and the Cell Cycle p34^{cdc2} Kinase in vitro. **J. Biol. Chem.** 2 -10

25. McCabe, P.C. is, P., McCormick, F., and Innis, M. A. 1992 Functiona 1^{rap1A} and Components of the Budding pathway of *Sacc* s c **Mol. Cell. Biol.** 12, 4084-4092.

26. Rubinfeld, B., ., Conroy,L., Clark, R., McCormick, F. and **Polakis, P.** 199 r GAP Catalytic Domain and Sites of Phosphorylation **Mol. Cell . Biol.** 12, 4634-4642.

27. Ando, S., K raoka, T., Nishiyama, T., Mizuno, T., Asada, M., Nun ura, Y., **Polakis, P.**, McCormick, F. and Takai, Y. 1992 P sing of rac p21s is important both for their interaction with proteins and for their activation of NADPH oxidase. **J. Biol** 13.

28. Janoueix-Le vitian, A. and deGunzberg, J. 1992 Regulation of th as-related rap2 protein. **Biochem. Biophys.** Res.

29. Polakis, P. e rap1/Krev-1 Protein. in GTP-binding Proteins: the *ra* le and F. McCormick, eds.) 445-452.

30. Polakis, P. Structural requirements for the interaction of p21^{ras} with G d its bological effector target. **J. Biol Chem.** 268, 91

31. Rubinfeld, B. ler, O., Chamberlain, S., Maslarz, F., Munemitsu, S. ciation of the APC gene product with beta- catenin. S

32. Weiss, J., R McCormick, F. Cavenee, W. A. and Arden, K. 1993 The g ase activating protein (rap1GAP) maps to chromosome 1p **Cell Genet.** 66, 18-21.

33. Sato, K. Y., ., Fasching, C. L., McCormick, F. and Stanbridge, E. mor suppressor acitvity of the K-rev gene in human tumor cel , 552-559.

34. Janoueix-L bbelem, G., Tavitian, A., **Polakis, P.** and DeGunzburg, rap1GAP during the cell cycle. **Biochem. Biophys.** Res.

35. Munemitsu, ., Albert, I., Rubinfeld, B., **and Polakis, P.** 1994 The APC with microtubules in vivo and affects their assembly in vitr 3-3681.

36. Rubinfeld, B. Purification of baculovirus produced rap1GAP. Meth

37. Polakis, P. 1 PC gene and their implications for protein structure and fur. ıs in Genetics and Development 5, 66-71

38. Rubinfeld, B., unemitsu, S. and **Polakis P.** 1995 The APC protein and but independent complexes with α-catenin, β-catenin and P. ı. 270, 5549-5555

39. Munemitsu, Rubinfeld, B., and **Polakis, P.** 1995 Regulation of int ls by the APC tumor suppressor gene. **Proc. Natl. Aca**

40. Lock, P., Fu icCormick, F. and Courtneidge, S. A. 1996 The human p62 nd not the rasGAP-associated p62 protein. **Cell** 84, 23-24.

41. Papkoff, J., 3. and **Polakis, P.** 1996 Wnt-1 regulates free pools of cat -catenin complexes. **Mol. Cell. Biol.** 16, 2128-2134.

42. Rubinfeld, E ı, C., Munemitsu, S. and **Polakis, P.** 1996 Binding of GSK3 complex and regulation of complex assembly. Scie

43. Munemitsu, J. and **Polakis, P.** 1996 Deletion of amino-terminal structure vivo and promotes the hyperphosphoryl suppressor protein. **Mol. Cell. Biol.**16, 4088-4094.

44. Hart, M. J., C and **Polakis P.** 1996 IQGAP1, a calmodulin bindi related domain, is a potential effector for cdc42Hs. EMBC

45. Nathke, I. S.. P., Sellin, J. and Nelson, W. J. 1996 The adenomatous p suppressor protein is localized to plasma membrane sites al cell migration. **J. Cell. Biol.** 134, 165-180.

46. Hart, M. J., C Qui, R-G., McCabe, P., **Polakis, P.** and Bollag, G. 1996 guanine nucleotide exchange factor for the rho GTPase. J.

47. Thomas JE, owski M, Beckmann RP, and **Polakis P.** 1996 Subcellula s of apparent 180-kDa and 220-kDa proteins of the b y gene, BRCA1. **J. Biol. Chem.** 1996 271, 28630-286

48. Hayashi, S., Polakis, P., Wieschaus, E., and Levine, A. 1997 A Dros r suppressor adenomatous polyposis coli

down-regulates β
regulation of arm

xpression is not essential for the
. Sci. 94, 242-247.

49. Vleminckx, K.,
tumor suppressor
136, 411-420.

, P. and Gumbiner, B. 1997 The APC
axis in Xenopus embryos. J. Cell. Biol.

50. Rubinfeld, B.
1997 Stabilizati
275, 1790-1792.

l., Albert, I., Porfiri, P. and Polakis, P.
defects in melanoma cell lines. Science

51. Polakis, P.
Biochem. Biophy

cis coli (APC) tumor suppressor. 1997
147.

52. Rubinfeld, B.
β-catenin regul
structure due to c
4630.

emitsu, S., and Polakis, P 1997 Loss of
ressor protein correlates with loss of
ns of the gene. Cancer Res. 57, 4624-

53. Porfiri, E., Ru
1997 Induction o
β-catenin. Onc

anes. K., Waterman, M., and Polakis, P.
plex by wnt-1 and transforming mutants of

54. Thomas JE,
Induction of phos
Cell Growth Diff

ubinfeld B, and Polakis P., 1997
uring the cell cycle and after DNA damage.

55. Hart, M., de
regulation of β-c
polyposis coli (A
Current Biology

ubinfeld, B., and Polakis P., 1998 Down
its association with the adenomatous
atenin and glycogen synthase kinase 3β.

56. Polakis, P.
Genetics and D

ation of β-catenin. Current Opinions in

57. Matt Hart,
Herve Durand,
Benarous and
phosphorylated
207-10.

Lassot, Iris Albert, Rico del los Santos,
binfled, Florence Margottin, Richard
ox protein β–TrCP associates with
activity in the cell. Current Biology 9,

58. Howard C.
and Lynn M. Ma
β–catenin trans

leton, Bonnee Rubinfeld, Paul Polakis
roteinase matrilysin is a target of
s. Oncogene 18, 2883-91.

59. Meng J, Glic
Galpha(z) and
17, 36663-9

1999 Functional interaction between
form of cellular cross-talk. J Biol Chem.

60. Vijayasurian ... Paul Polakis and Steve Byers 1999 The ubiquitin-proteo... kinase activity modulate APC mediated regulation of β-c... Biol. Chem. 274(23):16641-5.

61 Polakis P, H... Ru... 999 Defects in the regulation of beta-catenin in colorectal car... ...70, 23-32

62 Shen Z, Bat... ..., Schlessinger J, Lydon NB, Moran MF. 1999 Evidence ...ding and phosphorylation of Sam68 by Src. Oncogene. ...

64. Thomas GM... ...ke I, Polakis P, Cohen P. 1999 A GSK3- bindin... ...fectively inhibits the GSK3-catalysed phosphorylatio... ...EBS Lett. 458, 247-51.

65. Peifer M, P... ...ling in oncogenesis and embryogenesis--a look outside the ... ...606-9.

66. Polakis P. ... ...ncer. Genes Dev;14, 1837-1851.

67. Spink KE, P... ...ctural basis of the Axin-adenomatous polyposis coli in... ...0-2279.

68. Szeto, W., J... ...infeld, B., Hollingshead, P.G., Fong, S.E., Dugger, D.L., P... ...Vong, T.A., Grimaldi, J.C., Corpuz, R.T., Singh J.S., Fran... ...owley, C.W., Schwall, R.H., Eberhard, D.A., Rastelli, L., P... ...2001 Overexpression of the Retinoic Acid-Responsive Ge... ...and its Synergistic Induction by Wnt-1 and Retinoic Acid.

69. Rubinfeld B, ...xin dependent phosphorylation of the adenomatous p... ...by casein kinase 1 epsilon. J Biol Chem 276, 39037-39...

70. Polakis P. ... ...kin a catenin. Cell 2001 105, 563-566.

71. Tice DA, S... ...ivation of the Wnt Pathway Interferes withSerum Re... ...ption of Immediate Early Genes. J Biol. Chem. 277, 61...

72. Tice DA, S... ..., Fong SE, Dugger DL, Winer J,

Williams PM, Wi... ...H, Pennnica D, **Polakis P. 2002**
Synergistic activa... ...wnt-1 signaling and retinoic acid revealed
by gene expressio... ...n. 277,14329-14335.

73. Polakis, P. 2... ...vnt'er of disconnect. **Curr. Biol. 12, R499.**

74. Mao,W. , Lu... ...n; C., Crowley, C., Chui, C., Franz, G.,
Senter, P., Koc... ...EphB2 as a therapeutic antibody drug
target for the tr... ...e . Cancer Res. 64, 781-788.

75. Shibamoto, ... M., Polakis, P. 2003 A Blockade in Wnt
signaling is activ... ...diction of F9 teratocarcinoma cells. **Exp.**
Cell Res. 29211-

76. Zhang Y, Eber... , Wu TD, Zhou Y, Watanabe C, Luoh SM, **Polakis P,**
Hillan KJ, Woo... ...-quantitative gene expression profiling in normal and
cancer tissues.

Genom...-wide Stu... of Gene Copy Numbers, Transc...ipts, ...tein Levels in Pairs of Non-in...asiv...asive Human Transitional Cell Ca...cin

Torben F. Z... ...oft‡§, Th...ma... ...n¶, Frederic M. Waldman‖, Hans Wolf**, and Julio E. ...lls‡‡

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Received, Se... 26, 200... 2001

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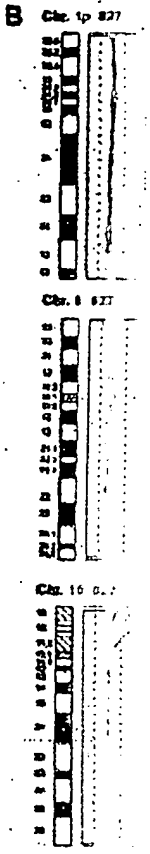
...eristic phenomenon at both the transcription and translation levels. ...ation in ... High throughput array studies of the breast cancer cell line ...oth the BT474 has suggested that there is a correlation between ...known DNA copy numbers and gene expression in highly amplified ...have at- areas (2), and studies of individual genes in solid tumors ...tempted to ... have revealed a good correlation between gene dose and ...ination mRNA or protein levels in the case of c-erb-B2, *cyclin d1*, ...hybrid- *ems1*, and N-myc (3–5). However, a high cyclin D1 protein ...onitor- expression has been observed without simultaneous am- ...olution plification (4), and a low level of c-myc copy number in- ...owed crease was observed without concomitant c-myc protein ...cases overexpression (6). ...this ef- ...o com- In human bladder tumors, karyotyping, fluorescent *in situ* ...(13 of hybridization, and comparative genomic hybridization (CGH)[1] ...ain have revealed chromosomal aberrations that seem to be ...ign- characteristic of certain stages of disease progression. In the ...land, case of non-invasive pTa transitional cell carcinomas (TCCs), ...de- this includes loss of chromosome 9 or parts of it, as well as ...gels loss of Y in males. In minimally invasive pT1 TCCs, the fol- ...and lowing alterations have been reported: 2q−, 11p−, 1q+, ...ased 11q13+, 17q+, and 20q+ (7–12). It has been suggested that ...ood these regions harbor tumor suppressor genes and onco- ...and genes; however, the large chromosomal areas involved often ...ons, contain many genes, making meaningful predictions of the ...lar functional consequences of losses and gains very difficult.

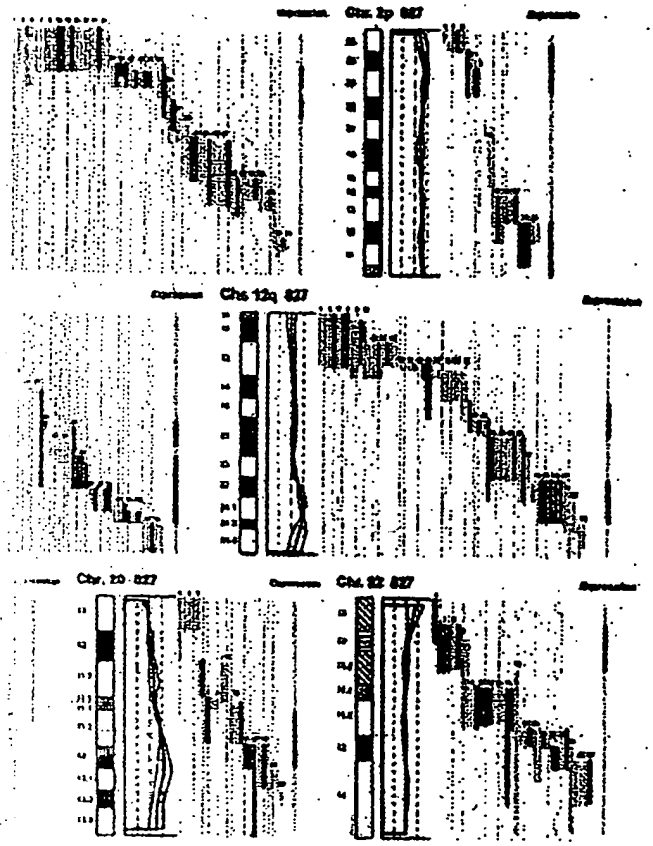
In this investigation we have combined genome-wide technology for detecting genomic gains and losses (CGH) with gene expression profiling techniques (microarrays and proteomics) to determine the effect of gene copy number on transcript and protein levels in pairs of non-invasive and invasive human bladder TCCs.

EXPERIMENTAL PROCEDURES

Material—Bladder tumor biopsies were sampled after informed consent was obtained and after removal of tissue for routine pathology examination. By light microscopy tumors 335 and 532 were staged by an experienced pathologist as pTa (superficial papillary),

[1] The abbreviations used are: CGH, comparative genomic hybridization; TCC, transitional cell carcinoma; LOH, loss of heterozygosity; PA-FABP, psoriasis-associated fatty acid-binding protein; 2D, two-dimensional.





1—*continued*

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labeled reference DNA (200 ng), and human Cot-1 DNA (20 μg) were denatured at 37 °C for 5 min and applied to denatured normal metaphase slides. Hybridization was at 37 °C for 2 days. After washing, the slides were counterstained with 0.15 μg/ml 4,6-diamidino-2-phenylindole in an anti-fade solution. A second hybridization was performed for all tumor samples using fluorescein-labeled reference DNA and Texas Red-labeled tumor DNA (inverse labeling) to confirm the aberrations detected during the initial hybridization. Each CGH experiment also included a normal control hybridization using fluorescein- and Texas Red-labeled normal DNA. Digital image analysis was used to identify chromosomal regions with abnormal fluorescence ratios, indicating regions of DNA gains and losses. The average green:red fluorescence intensity ratio profiles were calculated using four images of each chromosome (eight chromosomes total) with normalization of the green:red fluorescence intensity ratio for the entire metaphase and background correction. Chromosome identification was performed based on 4,6-diamidino-2-phenylindole banding patterns. Only images showing uniform high intensity fluorescence with minimal background staining were analyzed. All centromeres, p arms of acrocentric chromosomes, and heterochromatic regions were excluded from the analysis.

RESULTS

Comparative Genomic Hybridization—The CGH analysis identified a number of chromosomal gains and losses in the

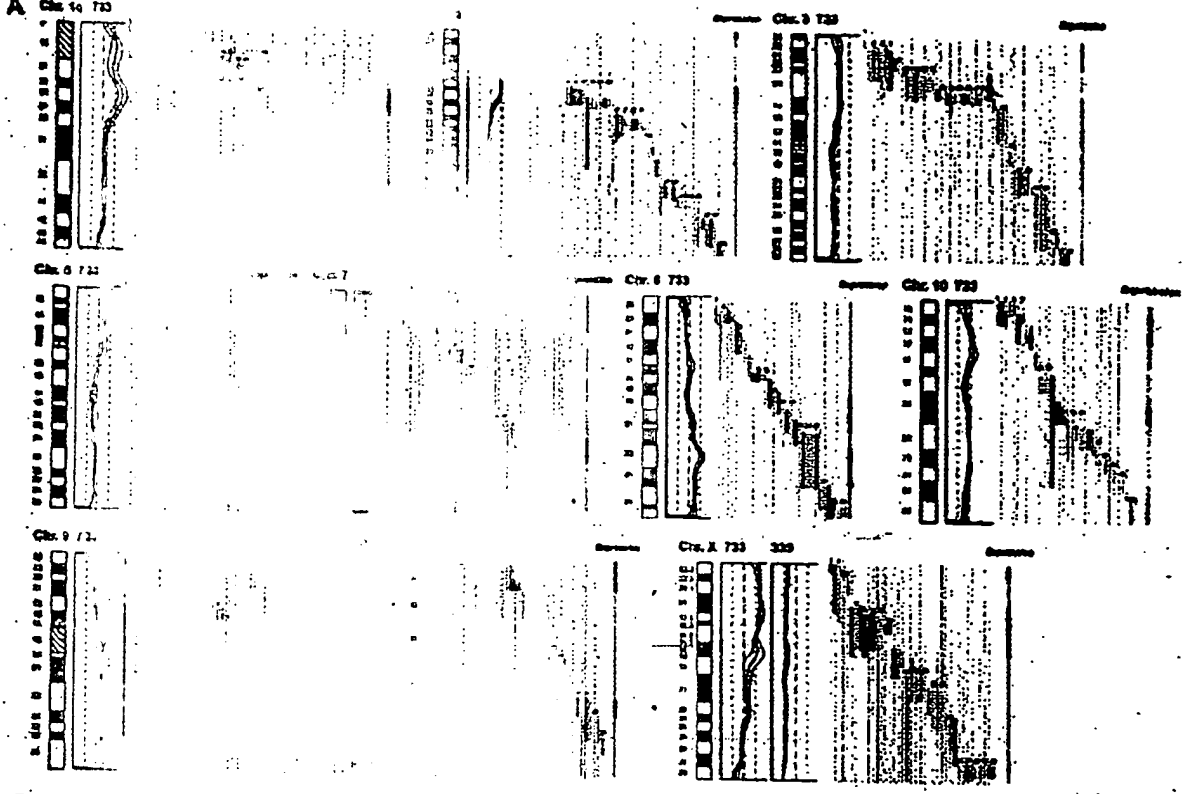

FIG. 1. ... expression ... compared ... counterpart ... (left). The ... deviation ... 0.5 (left) ... profile of ... running num... the gene, a... increase (b... in expressi... down-regul... another. It ... determinat...

...om left to right are chromosome (Chr.), CGH profiles, gene location and ...ong the chromosome. A, expression of mRNA in invasive tumor 733 as ...ession of mRNA in invasive tumor 827 compared with the non-invasive ...tumor DNA and normal DNA is shown along the length of the chromosome ...chromosomes and is surrounded by thin curves indicating one standard ...1 (no change), and the vertical lines next to it (dotted) indicate a ratio of ...335 used for comparison showed alterations in DNA content, the ratio ...or profile. The colored bars represents one gene each, identified by the ...t www.MDL.DK/sdata.html). The bars indicate the purported location of ...the invasive tumor compared with the non-invasive counterpart; >2-fold ...e). The bar to the far right, entitled Expression shows the resulting change ...t half of the genes were up-regulated (black), at least half of the genes ...d (orange). If a gene was absent in one of the samples and present in ...of was chosen as this corresponded to one standard deviation in a double ...regions were excluded from data analysis.

grade I and ... (invasive ... staged ac... mRNA ... were embe... solution a... RNAzol B ... poly(A)+ fr... mRNA kit; ... cRNA Pro... The first and ... SuperScript ... turer's instr... polymerase ... GAscript ...

UTP (Enzo) was used, together with unlabeled NTPs in the reaction. Following the *In vitro* transcription reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen).

Array Hybridization and Scanning—Array hybridization and scanning was modified from a previous method (13). 10 μg of cRNA was fragmented at 94 °C for 35 min in buffer containing 40 mM Tris acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc. Prior to hybridization, the fragmented cRNA in a 6× SSPE-T hybridization buffer (1 M NaCl, 10 mM Tris, pH 7.6, 0.005% Triton), was heated to 95 °C for 5 min, subsequently cooled to 40 °C, and loaded onto the Affymetrix probe array cartridge. The probe array was then incubated for 16 h at 40 °C at constant rotation (60 rpm). The probe array was exposed to 10 washes in 6× SSPE-T at 25 °C followed by 4 washes in 0.5× SSPE-T at 50 °C. The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate, 10 μg/ml (Molecular Probes) in 6× SSPE-T

TABLE I

Correlation [illegible] alter[illegible] [illegible]ed by CGH and by expression monitoring

Top, CGH [illegible] independent [illegible] (CGH alterations — what expression ratio was found); bottom, altered expression used as independent [illegible] that CGH alteration was found).

CGH altera[illegible]	Tumor 733 vs. 532 Expression change clusters	Concordance	CGH alterations	Tumor 827 vs. 532 Expression change clusters	Concordance
13 G[illegible]	[illegible]	[illegible]	10 Gain	8 Up-regulation 0 Down-regulation 2 No change	80%
10 Los[illegible]	[illegible]-regulation [illegible]-regulati[illegible] [illegible]	[illegible]	12 Loss	3 Up-regulation 2 Down regulation 7 No change	17%

Expression c[illegible]	Tumor 733 vs. 532 CGH alterations	Concordance	Expression change clusters	Tumor 827 vs. 532 CGH alterations	Concordance
16 Up-[illegible]	[illegible]	[illegible]	17 Up-regulation	10 Gain 5 Loss 2 No change	59%
21 Down-[illegible]	[illegible]	[illegible]	9 Down-regulation	0 Gain 3 Loss 6 No change	33%
15 No c[illegible]	[illegible] [illegible]ain[illegible] [illegible]os[illegible]	[illegible]	21 No change	1 Gain 3 Loss 17 No change	81%

two invasi[illegible] the two no[illegible] 532) show[illegible] and Y−, res[illegible] (1q22-24+, [illegible]14[illegible] 9q34+, 11[illegible] 13+ [illegible] ical for thei[illegible] some of [illegible] losses devi[illegible] and the a[illegible] In the cas[illegible] changes, [illegible] content, [illegible] 20q12 in T[illegible] *mRNA* [illegible] mRNA lev[illegible] 733) were [illegible] (TCCs 53[illegible] ments in [illegible] 532, res[illegible] arrays to [illegible] proximat[illegible] were sea[illegible] chromoso[illegible] (1096) w[illegible] that way [illegible] DNA cop[illegible] vidual ch[illegible] For ea[illegible] the invasi[illegible] represen[illegible] accordin[illegible]

than 2-fold were regarded as informative (Fig. 1). The density of genes along the chromosomes varied, and areas containing only one gene were excluded from the calculations. The resolution of the CGH method is very low, and some of the outlier data may be because of the fact that the boundaries of the chromosomal aberrations are not known at high resolution.

Two sets of calculations were made from the data. For the first set we used CGH alterations as the independent variable and estimated the frequency of expression alterations in these chromosomal areas. In general, areas with a strong gain of chromosomal material contained a cluster of genes having increased mRNA expression. For example, both chromosomes 1q21-q25, 2p and 9q, showed a relative gain of more than 100% in DNA copy number that was accompanied by increased mRNA expression levels in the two tumor pairs (Fig. 1). In most cases, chromosomal gains detected by CGH were accompanied by an increased level of transcripts in both TCCs 733 (77%) and 827 (80%) (Table I, *top*). Chromosomal losses, on the other hand, were not accompanied by decreased expression in several cases, and were often registered as having unaltered RNA levels (Table I, *top*). The inability to detect RNA expression changes in these cases was not because of fewer genes mapping to the lost regions (data not shown).

In the second set of calculations we selected expression alterations above 2-fold as the independent variable and estimated the frequency of CGH alterations in these areas. As above, we found that increased transcript expression correlated with gain of chromosomal material (TCC 733, 69% and TCC 827, 59%), whereas reduced expression was often detected in areas with unaltered CGH ratios (Table I, *bottom*). Furthermore, as a control we looked at areas with no alter-

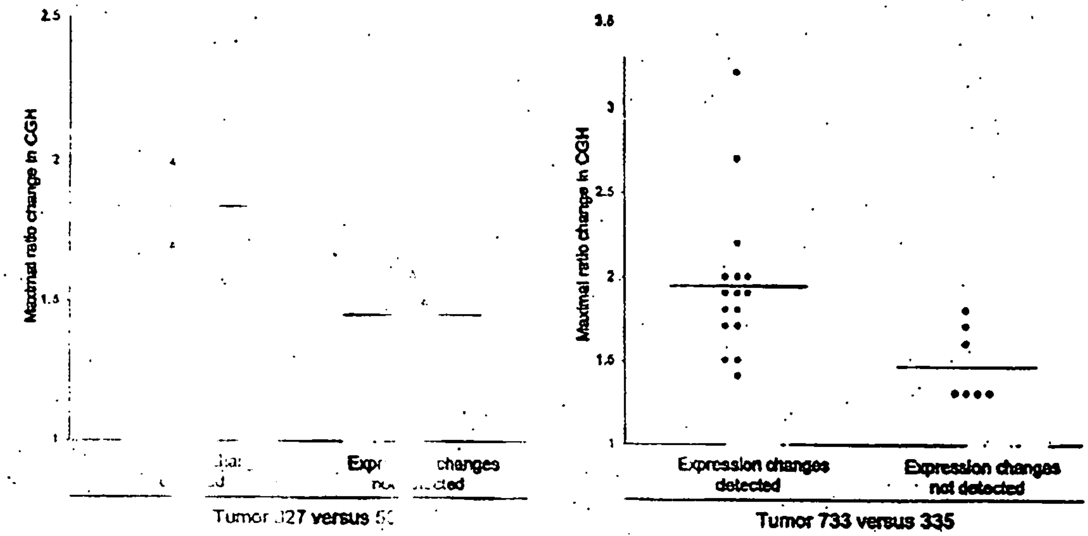

FIG. 2. Correlation ... maximum CGH ... ration and the ability to detect expression change by oligonucleotide array monitoring. The ab... shown as a numeric ... change in ratio between invasive tumors 827 (▲) and 733 (♦) and their non-invasive counterparts 532 and ... expression change ... taken from the *Expression* line to the *right* in Fig. 1, which depicts the resulting expression change ... somal region ... least half of the mRNAs from a given region have to be either up- or down-regulated to be scored as an ... All chromo... arms in which the CGH ratio plus or minus one standard deviation was outside the ratio value of one ... ed.

ation in expression. ... alteration was detected by CGH in most of these areas (... 733, ...% and TC... 81%; see Table I, *bottom*). ... the ability to obs... reduced or increased mRNA ... during to a ... chromosomal area ... extent ... by nu... changes, ... CGH a... regions sh... against the a... to detect a change in ... monitored by oligonucleotide arrays (... TCC 733 ... 0.015) and TCC 827 (... significant ... lation was observed between ... CGH ratio c... (reflecting the DNA copy nu... ions dete... the ... based tech... data were ... ined ... areas with ... used as i... ident... ables. The... with CG... the ... ratio devia... 1, *bottom* ... not at low... se data pro... reflect ... loss of an ... 50% red... in exp... sion level, ... nt for de... of ex... sion altera... ial mate... occu... much larg...

Microsat... of Mino... of ... es—In TCC ... mal are... iting ... amplificatio... owed by ... with ... mal CGH b... ssion (se... TCC ... 3 chromoso... ,21 and 3q34, ... 10q22). To ... results ... becau... of undetecte... aterial i... regio... or because of other non-structural mechanisms regulating transcription, we examined two microsatellites positioned at chromosome 1q25–32 and two at chromosome 2p22. Loss of heterozygosity (LOH) was found at both 1q25 and at 2p22 indicating that minor deleted areas were not detected with the resolution of CGH (Fig. 3). Additionally, chromosome 2p in TCC 733 showed a CGH pattern of gain/no change/gain of DNA that correlated with transcript increase/decrease/increase. Thus, for the areas showing increased expression there was a correlation with the DNA copy number alterations (Fig. 1A). As indicated above, the mRNA decrease observed in the middle of the chromosomal gain was because of LOH, implying that one of the mechanisms for mRNA down-regulation may be regions that have undergone smaller losses of chromosomal material. However, this cannot be detected with the resolution of the CGH method.

In both TCC 733 and TCC 827, the telomeric end of chromosome 11p showed a normal ratio in the CGH analysis; however, clusters of five and three genes, respectively, lost their expression. Two microsatellites (D11S1760, D11S922) positioned close to MUC2, IGF2, and cathepsin D indicated LOH as the most likely mechanism behind the loss of expression (data not shown).

A reduced expression of mRNA observed in TCC 733 at chromosomes 3q24, 11p11, 12p12.2, 12q21.1, and 16q24 and in TCC 827 at chromosome 11p15.5, 12p11, 15q11.2, and 18q12 was also examined for chromosomal losses using microsatellites positioned as close as possible to the gene loci

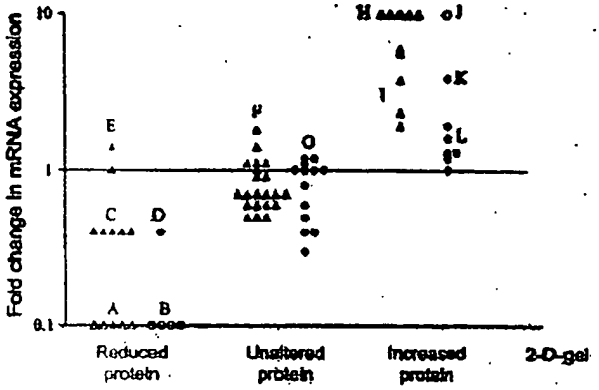

FIG. 4. Correlation between protein levels as judged by 2D-PAGE and transcript ratio. For comparison proteins were divided in three groups, unaltered in level or up- or down-regulated (*horizontal axis*). The mRNA ratio as determined by oligonucleotide arrays was plotted for each gene (*vertical axis*). ▲, mRNAs that were scored as present in both tumors used for the ratio calculation; Δ, mRNAs that were scored as absent in the invasive tumors (along *horizontal axis*) or as absent in non-invasive reference (*top* of figure). Two different scalings were used to exclude scaling as a confounder. TCCs 827 and 532 (▲Δ) were scaled with background suppression, and TCCs 733 and 335 (●○) were scaled without suppression. Both comparisons showed highly significant ($p < 0.005$) differences in mRNA ratios between the groups. Proteins shown were as follows: Group *A* (from *left*), phosphoglucomutase 1, glutathione transferase class-μ number 4, fatty acid-binding protein homologue, cytokeratin 15, and cytokeratin 13; *B* (from *left*), fatty acid-binding protein homologue, 28-kDa heat shock protein, cytokeratin 13, and calcyclin; *C* (from *left*), α-enolase, hnRNP B1, 28-kDa heat shock protein, 14-3-3-ε, and pre-mRNA splicing factor; *D*, mesothelial keratin K7 (type II); *E* (from *top*), glutathione *S*-transferase-π and mesothelial keratin K7 (type II); *F* (from *top* and *left*), adenylyl cyclase-associated protein, E-cadherin, keratin 19, calgizzarin, phosphoglycerate mutase, annexin IV, cytoskeletal γ-actin, hnRNP A1, integral membrane protein calnexin (IP90), hnRNP H, brain-type clathrin light chain-a, hnRNP F, 70-kDa heat shock protein, heterogeneous nuclear ribonucleoprotein A/B, translationally controlled tumor protein, liver glyceraldehyde-3-phosphate dehydrogenase, keratin 8, aldehyde reductase, and Na,K-ATPase β-1 subunit; *G*, (from *top* and *left*), TCP20, calgizzarin, 70-kDa heat shock protein, calnexin, hnRNP H, cytokeratin 15, ATP synthase, keratin 19, triosephosphate isomerase, hnRNP F, liver glyceraldehyde-3-phosphatase dehydrogenase, glutathione *S*-transferase-π, and keratin 8; *H* (from *left*), plasma gelsolin, autoantigen calreticulin, thioredoxin, and NAD+-dependent 15 hydroxyprostaglandin dehydrogenase; *I* (from *top*), prolyl 4-hydroxylase β-subunit, cytokeratin 20, cytokeratin 17, prohibitin, and fructose 1,6-biphosphatase; *J* annexin II; *K*, annexin IV; *L* (from *top* and *left*), 90-kDa heat shock protein, prolyl 4-hydroxylase β-subunit, α-enolase, GRP 78, cyclophilin, and cofilin.

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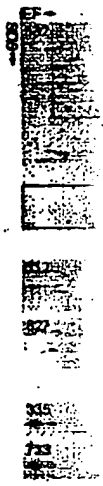
gradient, and having a known chromosomal location, were selected for analysis in the TCC pair 827/532. Proteins were identified by a combination of methods (see "Experimental Procedures"). In general there was a highly significant correlation ($p < 0.005$) between mRNA and protein alterations (Fig. 4). One gene showed disagreement between transcript alteration and protein alteration. Except for a group of cyto-



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keratins encoded by genes on chromosome 17 (Fig. 5) the analyzed proteins did not belong to a particular family. 28 well focused proteins whose genes had a know chromosomal location were detected in TCCs 733 and 335, and of these 19 correlated ($p < 0.005$) with the mRNA changes detected using the arrays (Fig. 4). For example, PA-FABP was highly expressed in the non-invasive TCC 335 but lost in the invasive counterpart (TCC 733; see Fig. 5). The smaller number of proteins detected in both 733 and 335 was because of the smaller size of the biopsies that were available.

11 chromosomal regions where CGH showed aberrations that corresponded to the changes in transcript levels also showed corresponding changes in the protein level (Table II). These regions included genes that encode proteins that are found to be frequently altered in bladder cancer, namely cytokeratins 17 and 20, annexins II and IV, and the fatty acid-binding proteins PA-FABP and FBP1. Four of these proteins were encoded by genes in chromosome 17q, a frequently amplified chromosomal area in invasive bladder cancers.

DISCUSSION

Most human cancers have abnormal DNA content, having lost some chromosomal parts and gained others. The present study provides some evidence as to the effect of these gains and losses on gene expression in two pairs of non-invasive and invasive TCCs using high throughput expression arrays and proteomics, in combination with CGH. In general, the results showed that there is a clear individual regulation of the mRNA expression of single genes, which in some cases was superimposed by a DNA copy number effect. In most cases, genes located in chromosomal areas with gains often exhibited increased mRNA expression, whereas areas showing losses showed either no change or a reduced mRNA expression. The latter might be because of the fact that losses most often are restricted to loss of one allele, and the cut-off point for detection of expression alterations was a 2-fold change, thus resulting at the border of detection. In several cases, how-

TABLE II
Genes with both mRNA and gene dose changes

TCC	CGH alteration	Transcript alteration[a]	Protein alteration
	Gain	Abs to Pres[a]	Increase
	Gain	3.9-Fold up	Increase
	Gain	3.8-Fold up	Increase
	Gain	5.6-Fold up	Increase
	Loss	10-Fold down	Decrease
	Gain	2.3-Fold up	Increase
	Gain	Abs to Pres	Increase
	Loss	2.5-Fold up	Decrease
	Gain	3.7-/2.5-Fold up[b]	Increase
	Gain	5.7-/1.8-Fold up	Increase
	Loss	2.5-Fold down	Decrease

...th TCCs 827 and 733 these are shown as 827/733.

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arm and that the use of cDNA microarrays for analysis of DNA copy number changes will reach a resolution that can resolve these changes, as has recently been proposed (2). The outlier data were not more frequent at the boundaries of the CGH aberrations. At present we do not know the mechanism behind chromosomal aneuploidy and cannot predict whether chromosomal gains will be transcribed to a larger extent than the two native alleles. A mechanism as genetic imprinting has an impact on the expression level in normal cells and is often reduced in tumors. However, the relation between imprinting and gain of chromosomal material is not known.

We regard it as a strength of this investigation that we were able to compare invasive tumors to benign tumors rather than to normal urothelium, as the tumors studied were biologically very close and probably may represent successive steps in the progression of bladder cancer. Despite the limited amount of fresh tissue available it was possible to apply three different state of the art methods. The observed correlation between [illegible] number and mRNA expression is remarkable when [illegible]ders that different pieces of the tumor biopsies were used [illegible] the different sets of experiments. This indicate that bladder tumors are relatively homogenous, a notion recently [illegible]d by CGH and LOH data that showed a remarkable [illegible] even between tumors and distant metastasis (10, 23). [illegible] few cases analyzed, mRNA and protein levels [illegible] a striking correspondence although in some cases [illegible] discrepancies that may be attributed to translational [illegible], post-translational processing, protein degradation [illegible] combination of these. Some transcripts belong to [illegible]ated mRNA pools, which are associated with few [illegible]ally inactive ribosomes; these pools, however, [illegible] rare (24). Protein degradation, for example, may [illegible] important in the case of polypeptides with a short [illegible] (e.g. signaling proteins). A poor correlation between [illegible] protein levels was found in liver cells as deter[illegible] arrays and 2D-PAGE (25), and a moderate correla[illegible] recently reported by Ideker *et al.* (26) in yeast. [illegible]singly, our study revealed a much better correlation [illegible] gained chromosomal areas and increased mRNA [illegible] between loss of chromosomal areas and reduced [illegible]els. In general, the level of CGH change determined [illegible] to detect a change in transcript. One possible [illegible] could be that by losing one allele the change in [illegible]vel is not so dramatic as compared with gain of [illegible] which can be rather unlimited and may lead to a [illegible] increase in gene copy number resulting in a much [illegible] on transcript level. The latter would be much [illegible] detect on the expression arrays as the cut-off point [illegible] at a 2-fold level so as not to be biased by noise on [illegible]. Construction of arrays with a better signal to noise [illegible] in the future allow detection of lesser than 2-fold [illegible] in transcript levels, a feature that may facilitate the [illegible] of the effect of loss of chromosomal areas on tran[illegible] [illegible]ysis.

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P., Mihatsch, M. J., and Waldman, F. M. (1995) c-myc copy number pairs in bladder cancer detected by fluorescence in situ hybridization. Am. J. Pathol. 146, 1131–1139

7. Richt... J., Jiang, F., Gorog, J. P., Sartorious, G., Egenter, C., Gasser, T. C., ...och, H., Mihatsch, M. J., and Sauter, G. (1997) Marked genetic differ... ces between stage pTa and stage pT1 papillary bladder cancer de... ted by comparative genomic hybridization. Cancer Res. 57, 2...–2864

8. ... J., Beffa, L., Wagner, U., Schraml, P., Gasser, T. C., Moch, H., ...sch, M. J., and Sauter, G. (1998) Patterns of chromosomal imbalances in advanced urinary bladder cancer detected by comparative genomic hybridization. Am. J. Pathol. 153, 1615–1621

9. ... Wöhr, G., Hautmann, R., Mattfeldt, T., Bruderlein, S., Moller, P., ... S., Hameister, H., Vogel, W., and Paiss, T. (1998) Chromosomal ... es during progression of transitional cell carcinoma of the bladder ... ification of the amplified interval on chromosome arm 8q. Genes Chromosomes Cancer 23, 167–174

10. H... R. M., Chu, L., Balazs, M., De Vries, S., Moore, D., Sauter, G., ...ell, P. R., and Waldman, F. M. (1998) Genetic alterations in primary ... cancers and their metastases. Cancer Res. 15, 3555–3560

11. ... Burger, H., Brinkschmidt, C., Bocker, W., Hertle, L., and Terpe, ... (199..) Chromosomal aberrations associated with invasion in pap... superficial bladder cancer. J. Pathol. 185, 345–351

12. ... Kwon, K. C., Ihm, C. H., Jeon, Y. M., Park, J. W., and Sul, C. K. ... Detection of genetic alterations in bladder tumors by comparative ... hybridization and cytogenetic analysis. Cancer Genet. Cyto... 110, 87–93

13. ... L., Byng, H., Mittmann, M., Ho, M. H., and Lockhart, D. J. (1997) ...wide expression monitoring in Saccharomyces cerevisiae. Nat. Biotechnol. 15, 1359–1367

14. ...sen, M., Sunde, L., Bolund, L., and Orntoft, T. F. (1999) Compari... three methods of microsatellite detection. Scand. J. Clin. Lab. ... 157–177

15. ... Østergaard, M., Basse, B., Celis, A., Lauridsen, J. B., Ratz, ... Andersen, L., Hein, B., Wolf, H., Orntoft, T. F., and Rasmussen, ... (199..) Loss of adipocyte-type fatty acid binding protein and other ... markers is associated with progression of human bladder ... cell carcinomas. Cancer Res. 56, 4782–4790

16. ... Ratz, G., Basse, B., Lauridsen, J. B., and Celis, A. (1994) In Cell ...: A Laboratory Handbook (Celis, J. E., ed) Vol. 3, pp. 222–230, ... Press, Orlando, FL

17. ... Tycko, B., and Sapienza, C. (1998) Monoallelic expression: ... only be one'. Trends Genet. 14, 435–438

18. ... A., Zuklys, S., Morel, C., Mizoguchi, E., Mobisson, K., ... S., Terhorst, C., Wishart, W., Golan, D. E., Bhan, A. K., and ... J. (1998) Monoallelic expression of the interleukin-2 locus. ... 76, 2118–2121

... C. I., and Bartolomei, M. S. (1999) Mechanisms of genomic ... Curr. Opin. Genet. Dev. 9, 164–170

20. ... Cui, H., He, L., Pfeifer, S., Malmikumpu, H., Jiang, S., ... P., and Hedborg, F. (1999) Mosaic allelic insulin-like growth 2 expression patterns reveal a link between Wilms' tumorigenesis ... genetic heterogeneity. Cancer Res. 59, 3889–3892

21. ...edborg, F., He, L., Nordenskjold, A., Sandstedt, B., Pfeifer-... S., and Ohlsson, R. (1997) Inactivation of H19, an imprinted and ... tumor repressor gene, is a preneoplastic event during Wilms' ... Cancer Res. 57, 4469–4473

... Saldanha, A. J., Styles, C. A., Lander, E. S., and Fink, G. R. ... regulation of gene expression. Science 285, 251–254

... Y..., Y., Markl, I. D., Hajyan, K., Jones, P. A., and Shibata, D. ... cancer genotype stability during clinical progression. ... Chromosomes Cancer 29, 26–32

... Schummer, M., Hood, L., and Morris, D. R. (1999) Messenger ... state: the second dimension of high-throughput expres... ...ing. Proc. Natl. Acad. Sci. U. S. A. 96, 10632–10636

... L., and Seilhamer, J. (1997) Comparison of selected mRNA and ... abundances in human liver. Electrophoresis 18, 533–537

... Thorsson, V., Ranish, J. A., Christmas, R., Buhler, J., Eng, J. K., ... R., Goodlett, D. R., Aebersold, R., and Hood, L. (2001) ... genomic and proteomic analyses of a systematically per... metabolic network. Science 292, 929–934

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Elizabeth Hym
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ABSTRACT

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INTRODUCTI

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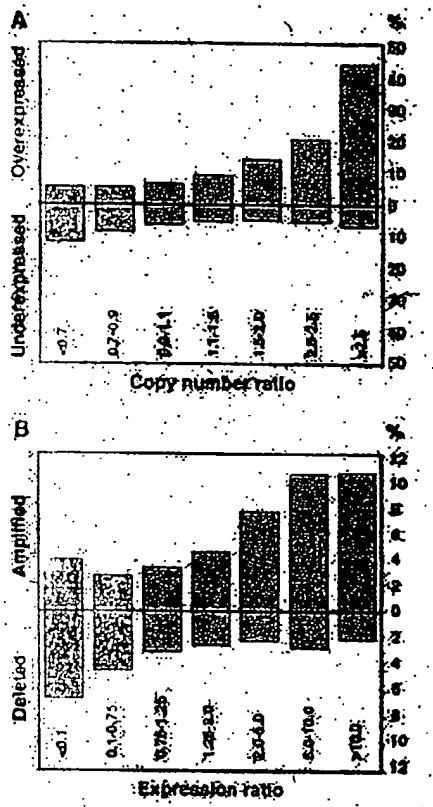
Patterns in Breast Cancer[1,2]

, Maija Wolf, Spyro Mousses, Ester Rozenblum,
, Olli-P. Kallioniemi, and Anne Kallioniemi[4]

Genetics Branch, National Human Genome Research Institute, NIH,
y of Cancer Genetics, Institute of Medical Technology, University of
ng Laboratory, Tampere University of Technology, FIN-33101 Tampere,
d Biomedicum Biochip Center, Helsinki University Hospital, Biomedicum



ig. 1. Impact of copy number on global gene expression levels. *A*, percentage of underexpressed genes (*Y axis*) according to copy number ratios (*X axis*). Genes used to define over- and underexpression were >2.184 (global upper 7% of ratios) and <0.4826 (global lower 7% of the expression ratios). *B*, percentage amplified and deleted genes according to expression ratios. Threshold values for amplification and deletion were >1.5 and <0.7.

recurrent regions of DNA amplification have been mapped in cancer by CGH[5] (9, 10). However, these amplicons are often poorly defined, and their impact on gene expression remains

hesized that genome-wide identification of those gene changes that are attributable to underlying gene copy rations would highlight transcripts that are actively in- the causation or maintenance of the malignant phenotype. y such transcripts, we applied a combination of cDNA and roarrays to: (*a*) determine the global impact that gene copy iation plays in breast cancer development and progression; ntify and characterize those genes whose mRNA expres-

[5] Abbreviations used: CGH, comparative genomic hybridization; FISH, fluo- hybridization; RT-PCR, reverse transcription-PCR.

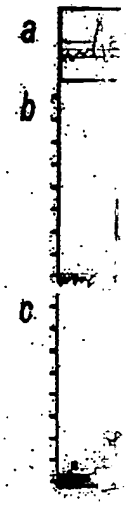


Fig. 2. Genome
line) across the entir
and *green line*, a ratio
of the cDNA clones al
copy number ratio of 1.
the next 5% of the ex
(underexpressed genes)
indicated with a *dashe*

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MATERIALS A

Breast Cancer C
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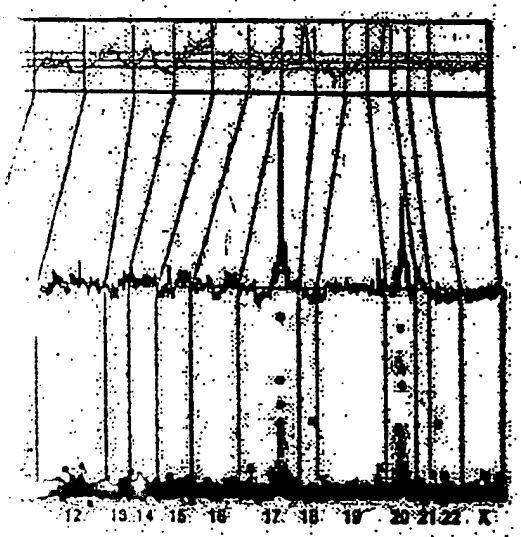

...mosomal CGH analysis of MCF-7. The copy number ratio profile (*blue*
The *black horizontal line* indicates a ratio of 1.0; *red line*, a ratio of 0.8;
...array. The copy number ratios were plotted as a function of the position
...moving median of 10 adjacent clones is shown. *Red horizontal line*, the
...ression ratios. The *bright red dots* indicate the upper 2%, and *dark red dots*,
...e lowest 2%, and *dark green dots*, the next 5% of the expression ratios
...bs are shown at the *bottom* of the figure, and chromosome boundaries are

...d from the analysis and were treated as missing values. The
...f fluorescence ratios were used to define cutpoints for increased/
...y number. Genes with CGH ratio >1.43 (representing the upper
...l ratios across all experiments) were considered to be amplified,
...h ratio <0.73 (representing the lower 5%) were considered to be

... Analysis of CGH and cDNA Microarray Data. To evaluate
...ct of copy number alterations on gene expression, we applied the
...tistical approach. CGH and cDNA calibrated intensity ratios were
...d and normalized using median centering of the values in each
...rmore, cDNA ratios for each gene across all 14 cell lines were
...d. For each gene, the CGH data were represented by a vector
...d 1 for amplification (ratio, >1.43) and 0 for no amplification.
...was correlated with gene expression using the signal-to-noise
We calculated a weight, w_g, for each gene as follows:

$$w_g = \frac{m_{g1} - m_{g0}}{\sigma_{g1} + \sigma_{g0}}$$

...and m_{g0}, σ_{g0} denote the means and SDs for the expression
...lified and nonamplified cell lines, respectively. To assess the
...ificance of each weight, we performed 10,000 random permu-
...label vectors. The probability that a gene had a larger or equal
...om permutation than the original weight was denoted by α. A
... indicates a strong association between gene expression and

... Localization of cDNA Clones and Amplicon Mapping. Each
...on the microarray was assigned to a Unigene cluster using the
...141.[6] A database of genomic sequence alignment information
...quences was created from the August 2001 freeze of the Uni-
...ome's Santa Cruz's GoldenPath database.[7] The chromosome and
...each cDNA clone were then retrieved by relating these data
...were defined as a CGH copy number ratio >2.0 in at least two
...of the two cell lines or a CGH ratio >2.0 in at least three
...in a single cell line. The amplicon start and end positions were

...ress: http://research.nhgri.nih.gov/microarray/downloadable_cdna.html.
...ess: www.genome.ucsc.edu.

Table 1 *Summ*

Location
1p13
1q21
1q22
3p14
7p12.1–7p11.2
7q31
7q32
8q21.11–8q21.1
8q21.3
8q23.3–8q24.[illegible]
8q24.22
9p13
13q22–q31.
16q22
17q11
17q12–q21.2
17q21.32–q21.3
17q22–q23.3
17q23.3–q24.3
19q13
20q11.22
20q13.12
20q13.12–q13.1
20q13.2–q13.32

extended to includ
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and 5′-GCGTCA
′).

RESULTS

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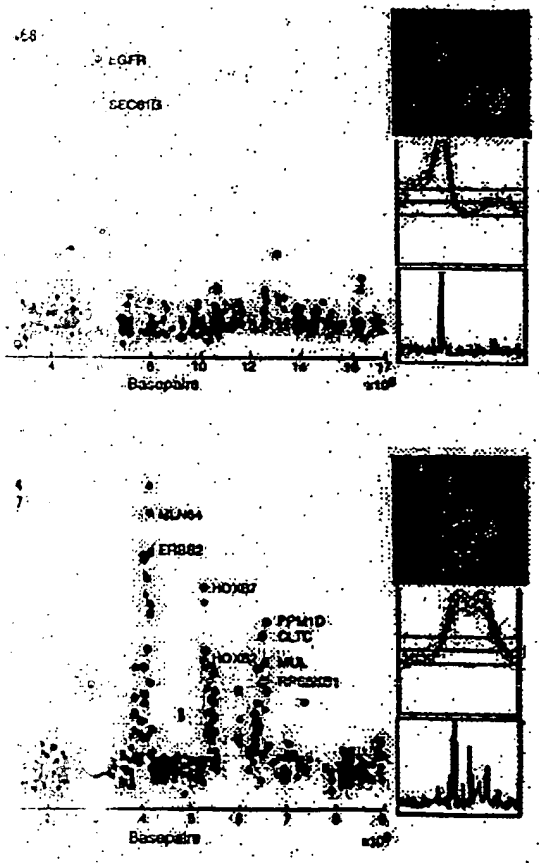
breast cancer
1). Several

validated, with 1q21, 17q12–q21.2, 17q22–q23, 20q13.1, .2 regions being most commonly amplified. Furthermore, ries of these amplicons were precisely delineated. In ad- el amplicons were identified at 9p13 (38.65–39.25 Mb), .3 (52.47–55.80 Mb).

dentification of Putative Amplification Target Genes. /CGH microarray technique enables the direct correla- y number and expression data on a gene-by-gene basis the genome. We directly annotated high-resolution with gene expression data using color coding. Fig. 2*C* most of the amplified genes in the MCF-7 breast cancer 1p13, 17q22–q23, and 20q13 were highly overex- view of chromosome 7 in the MDA-468 cell line *EGFR* as the most highly overexpressed and amplified 1–p12 (Fig. 3*A*). In BT-474, the two known amplicons nd 17q21–q23 contained numerous highly overex- es (Fig. 3*B*). In addition, several genes, including the genes *HOXB2* and *HOXB7*, were highly amplified in a un lescribed independent amplicon at 17q21.3. *HOXB7* tically a plified (as validated by FISH, Fig. 3*B*, *inset*) verexpre ed (as verified by RT-PCR, data not shown) UACC81 , and ZR-75-30 cells. Furthermore, this novel



tation of gen expression data on CGH microarray profiles. *A*, genes in the con in the M A-468 cell line are highly expressed (*red dots*) and include gene. *B*, se al genes in the 17q12, 17q21.3, and 17q23 amplicons in the cancer cel e are highly overexpressed (*red*) and include the *HOXB7* s labels an color coding are as indicated for Fig. 2*C*. *Insets* show GH profil for the corresponding chromosomes and validation of the number interphase FISH using *EGFR* (*red*) and chromosome 7 MDA-468 (*A*) and *HOXB7*-specific probe (*red*) and chro- cle (en) to BT-474 cells (*B*).

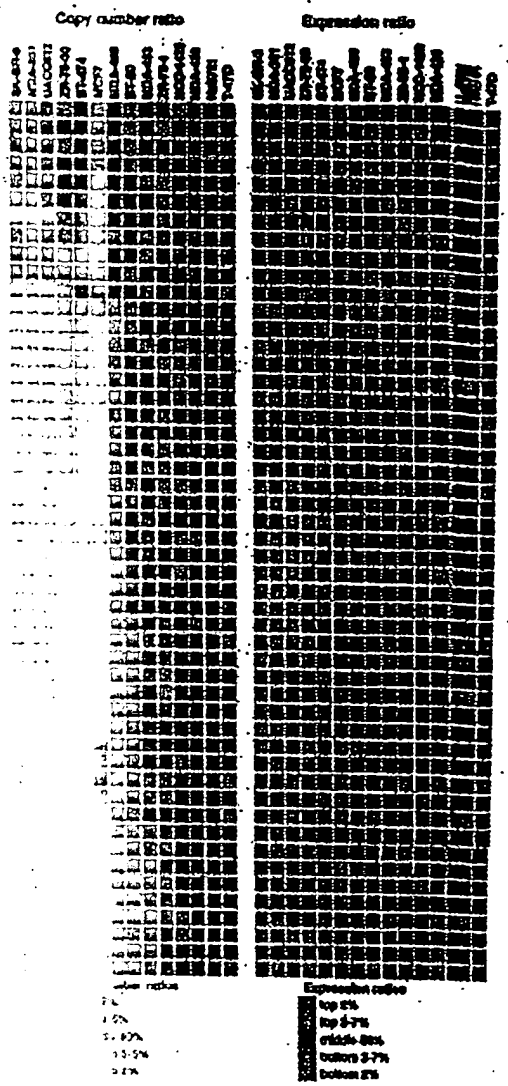


Fig. 4. List of
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ublications applying CGH[9] (9, 10), as well
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s. The effects of these somatic genetic
ion levels have remained largely unknown,
ve explored gene expression changes occur-
s (15, 19–21). Here, we applied genome-
to identify transcripts whose expression
to underlying gene copy number alterations

py number on gene expression patterns was
dramatic effects seen in the case of high-

[9] Internet address: ncbi.nlm.nih.gov/entrez.

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B7 amplification and poor patient prognosis. Overall, ustrate how the identification of genes activated by ation provides a powerful approach to highlight important role in cancer as well as to prioritize and tive targets for therapy development.

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Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., ... owning, J. R., Caligiuri, M. A., Bloomfield, C. D., and ... ssification of cancer: class discovery and class prediction ... ing. Science (Wash. DC), *286:* 531–537, 1999.

... B., Davis, R. E., Ma, C., Lossos, I. S., Rosenwald, A., ... ran, T., Yu, X., *et al.* Distinct types of diffuse large B-cell ... by gene expression profiling. Nature (Lond.), *403:* 503–511,

... tzer, P., Chen, Y., Jiang, Y., Seftor, E., Hendrix, M., Radmacher, M., ... akhini, Z., Ben-Dor, A., *et al.* Molecular classification of cutaneous ... noma by gene expression profiling. Nature (Lond.), *406:* 536–540,

... rlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., ... Johnsen, H., Akslen, L. A., *et al.* Molecular portraits of ... ure (Lond.), *406:* 747–752, 2000.

... e, T. R., Ghosh, D., Shah, R., Varambally, S., Kurachi, ... A., and Chinnaiyan, A. M. Delineation of prognostic ... er. Nature (Lond.), *412:* 822–826, 2001.

... bshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., ... M., Jeffrey, S. S., *et al.* Gene expression patterns of breast ... or subclasses with clinical implications. Proc. Natl. Acad. ... 9–10874, 2001.

... ckuber, J. A. The *HER-2/neu* oncogene: prognostic factor, predictive ... for therapy. Semin. Cancer Biol., *9:* 125–138, 1999.

... The epidermal growth factor receptor: from mutant oncogene in ... peutic target in human neoplasia. J. Clin. Oncol., *19:*

... M., Autio, K., Tarkkanen, M., Wolf, M., Monni, O., ... M. L., Tapper, J., Pere, H., El-Rifai, W., *et al.* DNA copy ... man neoplasms: review of comparative genomic hybrid- ... *152:* 1107–1123, 1998.

... alto Y. Online access to CGH data of DNA sequence ... J. Pathol., *157:* 689, 2000.

... wn, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., Chen, ... M. Use of a cDNA microarray to analyze gene expression ... Nat. Genet., *14:* 457–460, 1996.

... d Brown, P. O. A DNA microarray system for analyzing ... ing two-color fluorescent probe hybridization. Genome

... Chen, Y., Dougherty, E. R., Baxevanis, A., Meltzer, P. S., ... tion analysis by cDNA microarrays. *In:* F. J. Livesey and ... Genomics, pp. 113–137. Oxford: Oxford University

... Alizadeh, A. A., Eisen, M. B., Pergamenschikov, A., ... Botstein, D., and Brown, P. O. Genome-wide analysis ... ges using cDNA microarrays. Nat. Genet., *23:* 41–46,

... Mousses, S., Kononen, J., Sauter, G., Heiskanen, M., ... Y., Bittner, M. L., and Kallioniemi, A. Comprehensive ... ession profiling of the 17q23 amplicon in human breast ... ci. USA, *98:* 5711–5716, 2001.

... R., and Bittner, M. L. Ratio-based decisions and the ... A microarray images. J. Biomed. Optics, *2:* 364–374,

... Kononen, J., Bubendorf, L., Chen, Y., Bittner, M. L., ... r, C., Sauter, G., *et al.* Detecting activation of ribosomal ... mentary DNA and tissue microarray analysis. J. Natl. ... 2000.

... G., Grigoryan, A., Sauter, G., and Kallioniemi, A. ... uorescence *in situ* hybridization on tissue microarrays.

... Monni, O., and Kallioniemi, A. New amplified and ... vered in the ERBB2 amplicon in breast cancer by cDNA ... *61:* 8235–8240, 2001.

... M., Flohr, P., Gordon, T., Maillard, K., Giddings, I., ... Campbell, C., Shipley, J., Wooster, R., and Cooper, ... ed and expressed genes in breast cancer by comparative ... s of randomly selected cDNA clones. Genes Chromo- ... 2002.

... D., Vakkari, M. L., Kokkola, A., Moskaluk, C., Frierson, ... S., Kallioniemi, A., and El-Rifai, W. Targets of gene ... ssion at 17q in gastric cancer. Cancer Res., *62:* 2625–

... J., Dai, H., Jones, A. R., Meyer, M. R., Slade, D., ... T. R., Kidd, M. J., Friend, S. H., and Marton M. J.

Widespread ...
Genet., 25: 33 ...
23. Virtaneva, K., ...
Bloomfield, C. ...
fundamental bi...
and normal cyt...
24. Phillips, J. L., Ha...
H., Perullo, J. R. ...
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expression pro...
8143–8149, 20...
25. Bärtlind, M., T...
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to high-level ...
372–376, 19...
26. Tanner, M. ...
Kowbel, D., ...
Independent ...

... of chromosome 20 in human breast cancer. Cancer Res., 56: ...
... A., Candila, M., and Boncinelli, E. Homeobox genes and cancer. ... , 1999.
... ...ella, A., and Boncinelli, E. Homeobox genes in normal and ... Physiol., 188: 161–169, 2001.
... ...eccia, E., Mattia, G., Stoppacciaro, A., Parmiani, G., Peschle, ... P. HOXB7 constitutively activates basic fibroblast growth ...ol. Cell. Biol., 16: 4842–4851, 1996.
... Meccia, E., Mattia, G., Peschle, C., and Colombo, M. P. ...3 breast carcinoma cell line with the HOXB7 gene induces ... ...ases cell proliferation and reduces growth factor dependence. ... 89, 1998.
... ...Meccia, E., Bottaro, L., Parenza, M., Stoppacciaro, A., Peschle, ... HOXB7: a key factor for tumor-associated angiogenic switch. ... -6539, 2001.
... Montz, F. J., Seidman, J. D., Kurman, R. J., and Roden, R. B. ...ied tumor antigen encoded by a homeobox gene promotes ...elial cells. Proc. Natl. Acad. Sci. USA, 98: 4060–4065, 2001.

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l*†, Stefanie S. Jeffrey‡†, Per E. Lonning§¶,
k O. Brown†‖**

h and †Howard Hughes Medical Institute, Stanford
pital, Montebello, N-0310 Oslo, Norway;
Department of Genetics and Lineberger

we have identified a significant impact of wide-
copy number alteration on the transcriptional
east tumors.

n
ethods

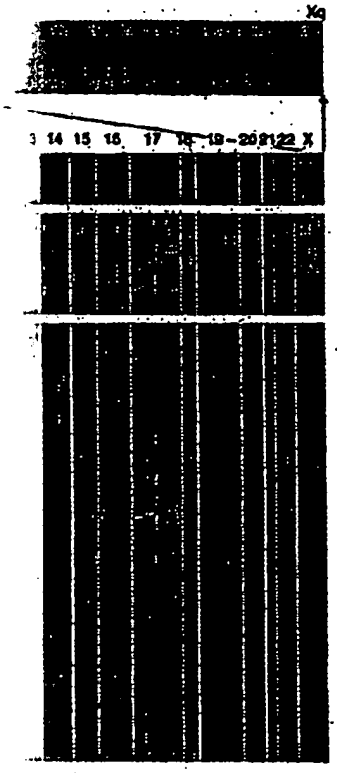
Cell Lines. Primary breast tumors were predominantly
termediate-grade, infiltrating ductal carcino-
than 50% being lymph node positive. The
r cells within specimens averaged at least 50%.
dual tumors have been published (8, 9), and
d in Table 1, which is published as supporting
the PNAS web site, www.pnas.org. Breast cancer
obtained from the American Type Culture
mic DNA was isolated either using Qiagen
columns, or by phenol/chloroform extraction
ol precipitation.

Microarray Hybridizations. Genomic DNA label-
ions were performed essentially as described
7), with slight modifications. Two micrograms
ed in a total volume of 50 microliters and the
gents were adjusted accordingly. "Test" DNA
cell lines) was fluorescently labeled (Cy5) and
human cDNA microarray containing 6,691
human genes (i.e., UniGene clusters). The
eled with Cy3) for each hybridization was nor-
yte DNA from a single donor. The fabrication
arrays and the labeling and hybridization of
ve been described (8).

Map Positions. Hybridized arrays were scanned
ner (Axon Instruments, Foster City, CA), and
(test/reference) calculated using SCANALYZE
at http://rana.lbl.gov). Fluorescence ratios
for each array by setting the average log
for all array elements equal to 0. Measure-
cence intensities more than 20% above back-
dered reliable. DNA copy number profiles
ficantly from background ratios measured in
NA control hybridizations were interpreted as
NA copy number alteration (see *Estimating
nd Fluorescence Ratios* in the supporting
indicated, DNA copy number profiles are
average (symmetric 5-nearest neighbors),
rrayed human cDNAs were assigned by

ve genomic hybridization.

hould be addressed at: Department of Pathology, Stanford
ne, CCSR Building, Room 3245A, 269 Campus Drive, Stanford,
ack1@stanford.edu.
, Hayward, CA 94545.



ofiles are illustrated for cell lines containing different
ferent cell line or tumor, and each column represents
om 1pter through Xqter. Moving average (symmetric
scale (indicated), such that red luminescence reflects
en poorly measured data). (*b*) Enlarged view of DNA
hromosomes.

Fig. 1. G
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nalysis of DNA from cell lines containing
f X chromosomes (Fig. 1*b*), as we did before
ne sensitivity of our method to detect single-
D), and 1.5- (47,XXX), 2- (48,XXXX), or
X) gains (also see Fig. 5, which is published
nation on the PNAS web site). Fluorescence
proportional to copy number ratios, which
estimated, in agreement with previous ob-
crous DNA copy number alterations were
breast cancer cell lines and primary tumors
n the tumors despite the presence of euploid
s; the magnitudes of the observed changes
r in the tumor samples. DNA copy-number
nd in every cancer cell line and tumor, and
omosome in at least one sample. Recurrent
py number gain and loss were readily iden-
fic, gains within 1q, 8q, 17q, and 20q were
roportion of breast cancer cell lines/tumors
7%, 100%/60%, and 90%/44%, respective-
within 1p, 3p, 8p, and 13q (80%/24%,
fic, and 70%/18%, respectively), consistent
genetic studies (refs. 2–4; a complete listing
vided in Tables 2 and 3, which are published
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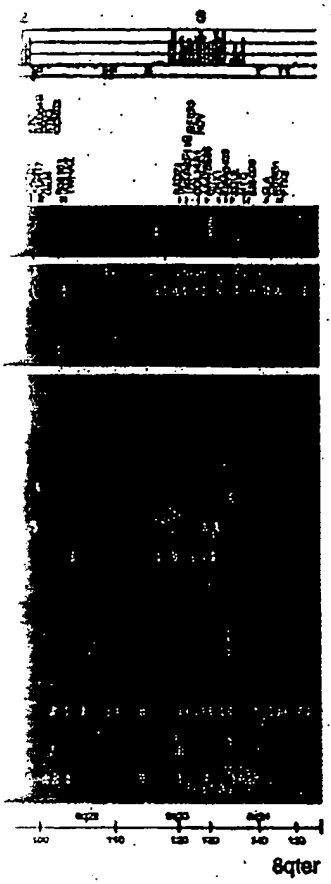

re illustrated for cell lines containing different numbers
rs are separately ordered by hierarchical clustering to
to chromosome 8 are ordered by position along the
lected genes are indicated with color-coded text (red,
d mRNA levels (observed in the majority of the subset
not represented on the microarray are indicated in the
for breast cancer cell line SKBR3. Fluorescence ratios

interval recurrently amplified in the tumors we
case, known or plausible candidate oncogenes
n description of these regions, as well as the
d regions on chromosomes 17 and 20, can be
nd 7, which are published as supporting
PNAS web site).
reast cancer cell lines and tumors (4 and 37,
a subset of arrayed genes (6,095), mRNA
tively measured in parallel by using cDNA
be parallel assessment of mRNA levels is
retation of DNA copy number changes. For
ly amplified genes that are also highly ex-
ngest candidate oncogenes within an ampli-
significantly, our parallel analysis of DNA
es and mRNA levels provides us the oppor-
global impact of widespread DNA copy
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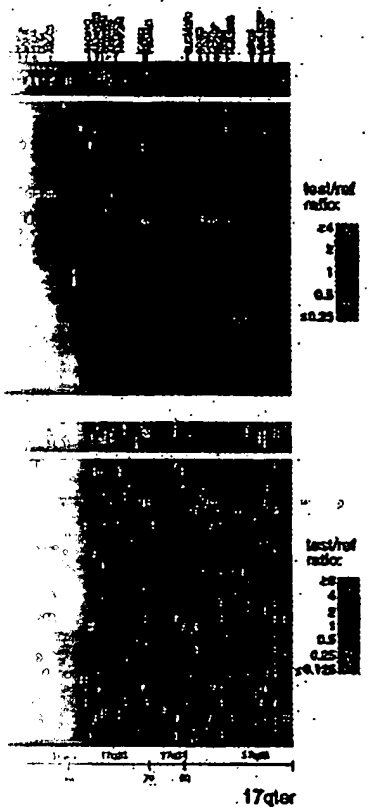
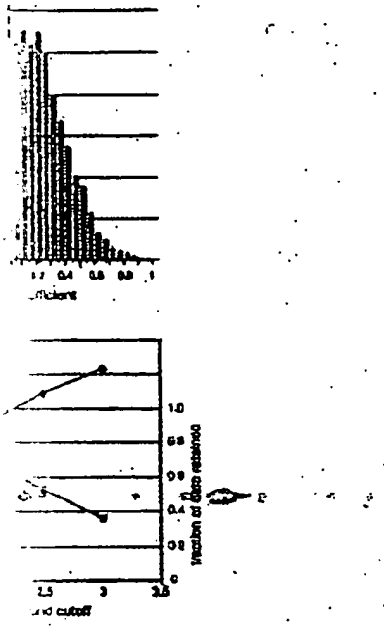


...alteration (*Upper*) and mRNA levels (*Lower*) ... by hierarchical clustering (*Upper*), and the ... 17, and for which both DNA copy number ... indicated in color-coded text (see Fig. 2 legend).

...nes and tumors, average mRNA levels ... copy number across all five classes, in a ... fashion (*P* values for pair-wise Student's ... ent classes: cell lines, 4×10^{-49}, 1×10^{-49}, ... tumors, 1×10^{-43}, $1^{5} \times 10^{-214}$, 5×10^{-41}, ...gression of the average log(DNA copy ...lass, against average log(mRNA level) ...verage, a 2-fold change in DNA copy ...ied by 1.4- and 1.5-fold changes in mRNA ...cer cell lines and tumors, respectively (Fig. ...ot shown). Second, we characterized the ...25 correlations between DNA copy num... ...ach across the 37 tumor samples (Fig. 4*b*). ...rrelations forms a normal-shaped curve, ...tedly shifted in the positive direction from ...tically significant, as evidenced in a plot ...ed correlations (Fig. 4*c*), and reflects a ...ce of DNA copy number alterations on ...; the highest correlations between DNA ...A level (the right tail of the distribution ...th amplified and deleted genes (data not ... a linear regression model to estimate the ... measured in mRNA levels among the 37



...ll lines (gray) and tumor samples (black), both ...tile) and averages (diamonds; Y-value error bars ...(tumor/normal ratio < 0.8), no change (0.8–1.2), ...ing averages between adjacent classes (moving ...umors). (b) Distribution of correlations between ...bserved versus expected correlation coefficients. ...The line of unity is indicated. (c) Percent variance ...ained (black line) and fraction of data retained ...toff values. Fraction of data retained is relative ... of variation in gene expression attributable to ...on of Variation in Gene Expression Attributable

...though the DNA microarrays used in our ...bias toward characterized and/or highly ...e we are examining such a large fraction ...imately 20% of all human genes), and ...ove, we are likely underestimating the ...opy number changes to altered gene ...ur findings are likely to be generalizable ...still be remarkable if only applicable to ...).

...euploidy has been shown to result in ...expression biases (13). Two recent ...amine the global relationship between ...gene expression in cancer cells. In ...ings, Phillips *et al.* (14) have shown that ...morigenicity in an immortalized pros-..., new chromosomal gains and losses ...ly significant respective increase and ... expression level of involved genes. In ...5) recently reported that in metastatic ... of genes within amplified regions were ...-fold) expressed, when compared with ...m. This report differs substantially from ...highly amplified genes in breast cancer ...creased expression. These contrasting ...thodological differences between the

of expressed genes, even within existing
ion data sets, may permit the inference
rration, particularly aneuploidy (where
averaged across large chromosomal
supporting information). Fifth, this
substantial portion of the phenotypic
tension, the heterogeneity in clinical
ts' tumors may be traceable to underly-
py number. Sixth, this finding supports
pread DNA copy number alteration in
beyond the amplification of specific
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umber alteration, and the concomitant
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ips in cell metabolism and physiology
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erapies that exploit specific or global
esslon in cancer.

ers of the P.O.B. and D.B. labs for helpful
Howard Hughes Medical Institute Physician
a portion of this work. P.O.B. is a Howard
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e National Institutes of Health, the Howard
the Norwegian Cancer Society, and the
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n, M., Jeffrey, S. S., *et al.* (2001) *Proc. Natl. Acad.*

l. Med. 75, 694–698.
., Birren, B., Nusbaum, C., Zody, M. C., Baldwin,
Doyle, M., FitzHugh, W., *et al.* (2001) *Nature*

hen, C., Waldman, F. & Gray, J. W. (1998) *Genes*
5–113.
J., Dai, H., Jones, A. R., Meyer, M. R., Slade, D.,
J. T. R., Kidd, M. J., *et al.* (2000) *Nat. Genet.* 25,

W., Wang, Y., Vassilli, J., Pavlovich, C., Padilla-
Ladimi, B. M., Grossfeld, G. D., Rivera, A., *et al.*
3–8149.
Wilson, K., Willis, J., Lutterbaugh, J., Nosrati, A.,
Reed, T. & Markowitz, S. (2002) *Cancer Res.* 62,

Segraves, R., Collins, C., Dairkee, S. H., Kowbel,
J. W. & Pinkel, D. (2000) *Nat. Genet.* 25,

tberg, P., Kraemer, A., Willer, A., Rausch, C. &
Natl. Acad. Sci. USA 94, 14506–14511.
. H. (1999) *Biochem. J.* 340, 621–630.

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Editorial:

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Refereed papers:

1. Gertler, A., Ashkenazi, A., and Madar, Z. Binding sites for human growth hormone and ovine and bovine prolactins in the mammary gland and liver of the lactating cow. *Mol. Cell. Endocrinol.* **34**, 51-57 (1984).
2. Gertler, A., Shamay, A., Cohen, N., Ashkenazi, A., Friesen, H., Levanon, A., Gorecki, M., Aviv, H., Hadari, D., and Vogel, T. Inhibition of lactogenic activities of ovine prolactin and human growth hormone (hGH) by a novel form of a modified recombinant hGH. *Endocrinology* **118**, 720-726 (1986).
3. Ashkenazi, A., Madar, Z., and Gertler, A. Partial purification and characterization of bovine mammary gland prolactin receptor. *Mol. Cell. Endocrinol.* **50**, 79-87 (1987).
4. Ashkenazi, A., Pines, M., and Gertler, A. Down-regulation of lactogenic hormone receptors in Nb2 lymphoma cells by cholera toxin. *Biochemistry Internatl.* **14**, 1065-1072 (1987).
5. Ashkenazi, A., Cohen, R., and Gertler, A. Characterization of lactogen receptors in lactogenic hormone-dependent and independent Nb2 lymphoma cell lines. *FEBS Lett.* **210**, 51-55 (1987).
6. Ashkenazi, A., Vogel, T., Barash, I., Hadari, D., Levanon, A., Gorecki, M., and Gertler, A. Comparative study on in vitro and in vivo modulation of lactogenic and somatotropic receptors by native human growth hormone and its modified recombinant analog. *Endocrinology* **121**, 414-419 (1987).
7. Peralta, E., Winslow, J., Peterson, G., Smith, D., Ashkenazi, A., Ramachandran, J., Schimerlik, M., and Capon, D. Primary structure and biochemical properties of an M2 muscarinic receptor. *Science* **236**, 600-605 (1987).
8. Peralta, E. Ashkenazi, A., Winslow, J., Smith, D., Ramachandran, J., and Capon, D. J. Distinont primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *EMBO J.* **6**, 3923-3929 (1987).
9. Ashkenazi, A., Winslow, J., Peralta, E., Peterson, G., Schimerlik, M., Capon, D., and Ramachandran, J. An M2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science* **238**, 672-675 (1987).

10. Pines, M., Ashkenazi, A., Cohen-Chapnik, N., Binder, L., and Gertler, A. Inhibition of the proliferation of Nb2 lymphoma cells by femtomolar concentrations of cholera toxin and partial reversal of the effect by 12-o-tetradecanoyl-phorbol-13-acetate. *J. Cell. Biochem.* 37, 119-129 (1988).
11. Peralta, E. Ashkenazi, A., Winslow, J. Ramachandran, J., and Capon, D. Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. *Nature* 334, 434-437 (1988).
12. Ashkenazi, A. Peralta, E., Winslow, J., Ramachandran, J., and Capon, D. Functionally distinct G proteins couple different receptors to PI hydrolysis in the same cell. *Cell* 56, 487-493 (1989).
13. Ashkenazi, A., Ramachandran, J., and Capon, D. Acetylcholine analogue stimulates DNA synthesis in brain-derived cells via specific muscarinic acetylcholine receptor subtypes. *Nature* 340, 146-150 (1989).
14. Lammare, D., Ashkenazi, A., Fleury, S., Smith, D., Sekaly, R., and Capon, D.